Acquired Resistance to Acetaminophen Hepatotoxicity is Associated with Induction of Multidrug Resistance-Associated Protein 4 (Mrp4) in Proliferating Hepatocytes

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Treatment with hepatotoxicants such as acetaminophen (APAP) causes resistance to a second, higher dose of the same toxicant (autoprotection). APAP induces hepatic mRNA and protein levels of the multidrug resistance-associated proteins (Mrp) transporters in mice and humans. Basolateral efflux transporters Mrp3 and Mrp4 are the most significantly induced. We hypothesized that upregulation of Mrp3 and Mrp4 is one mechanism by which hepatocytes become resistant to a subsequent higher dose of APAP by limiting accumulation of xeno-, endobiotics, and byproducts of hepatocellular injury. The purpose of this study was to evaluate Mrp3 and Mrp4 expression in proliferating hepatocytes in a mouse model of APAP autoprotection. Plasma and livers were collected from male C57BL/6J mice treated with APAP 400 mg/kg for determination of hepatotoxicity and protein expression. Maximal Mrp3 and Mrp4 induction occurred 48 h after APAP. Mrp4 upregulation occurred selectively in proliferating hepatocytes. Additional groups of APAP-pretreated mice were challenged 48 h later with a second, higher dose of APAP. APAP-pretreated mice had reduced hepatotoxicity after APAP challenge compared to those pretreated with vehicle. A more rapid recovery of glutathione (GSH) in APAP-pretreated mice corresponded with increases in GSH synthetic enzymes. Interestingly, mice pretreated and challenged with APAP had dramatic increases in Mrp4 expression as well as enhanced hepatocyte proliferation. Inhibition of hepatocyte replication with colchicine not only restored sensitivity of APAP-pretreated mice to injury, but also blocked Mrp4 induction. Mrp4 overexpression may be one phenotypic property of proliferating hepatocytes that protects against subsequent hepatotoxicant exposure by mechanisms that are presently unknown.

Key Words: acetaminophen; APAP; autoprotection; Mrp3; Mrp4; transporter; proliferation.

Acute or cumulative acetaminophen (APAP) overdose causes centrilobular hepatocyte degeneration and necrosis in experimental rodents and humans. APAP hepatotoxicity is the leading cause of drug-induced liver failure in the United States with well over 100,000 cases of APAP poisonings reported each year (Lee, 2004). A large portion of a therapeutic dose of APAP is metabolized in the liver by glucuronidation and sulfation and then excreted into the urine and bile. A small fraction of the dose is bioactivated by cytochrome P450 enzymes (isoforms Cyp1a2, 2e1, and 3a11) to the reactive electrophile, N-acetyl-*p*-benzoquinonimine (NAPQI) (Patten *et al.*, 1993). This metabolic intermediate reacts with glutathione (GSH) and the resulting conjugate is subsequently eliminated in the bile (Chen *et al.*, 2003). During overdose, glucuronidation and sulfation pathways become saturated and a greater proportion of APAP is bioactivated. Among other cellular events, once GSH stores are exhausted, remaining NAPQI binds thiol groups on cellular proteins (Jaeschke and Bajt, 2006; Jollow *et al.*, 1973). The net result is generation of mitochondrial dysfunction, oxidative stress, lipid peroxidation and hepatocyte degeneration and/or death (Jaeschke and Bajt, 2006).

In response to injury by APAP and other hepatotoxicants, there is a recovery phase in which normally quiescent hepatocytes enter the cell cycle in an attempt to replace necrotic cells (Zieve *et al.*, 1985). Replicating hepatocytes appear in the transition zone between necrotic and healthy cells in livers from APAP-treated mice (Bajt *et al.*, 2003). During this period of regeneration, rodents are resistant to a second challenge with a higher dose of the same hepatotoxicant, a phenomenon known as autoprotection (Thakore and Mehendale, 1991). Resistance to a different toxicant (heteroprotection) has also been observed (Chanda *et al.*, 1995; Yim *et al.*, 2006). Exposure to repeated doses of APAP similarly triggers hepatocyte proliferation and resilience to chemical injury (Dalhoff *et al.*, 2001; Shayiq *et al.*, 1999; Strubelt *et al.*, 1979). Interruption of hepatocyte replication by the antimitotic chemical colchicine restores sensitivity of the hepatotoxicant-pretreated rodents to subsequent toxicant challenge (Chanda *et al.*, 1995;

Shayiq *et al.*, 1999). The mechanism(s) underlying the resilience of proliferating hepatocytes to further toxicity is not completely known.

Compensatory changes in a number of metabolic and detoxification pathways have been proposed as potential mechanisms to explain auto- and heteroprotection. These include decreased bioactivation due to downregulation of Cyp450 enzymes (Pound and Lawson, 1975; Shayiq *et al.*, 1999; Yim *et al.*, 2006), zonal redistribution of Cyp450 enzymes (Shayiq *et al.*, 1999), changes in GSH turnover (Dalhoff *et al.*, 2001) and altered APAP covalent protein binding patterns (Shayiq *et al.*, 1999). Using the thioacetamide-APAP heteroprotection model, researchers have demonstrated that APAP bioactivation and GSH detoxification pathways do not fully account for protection (Chanda *et al.*, 1995). Thus, the mechanism(s) for resistance of proliferating hepatocytes is not well-established, and likely involves multiple cellular recovery pathways.

APAP-induced hepatotoxicity results in overexpression of efflux membrane transporters including some isoforms of the multidrug resistance-associated protein (Mrp) superfamily. Mrps are ATP-dependent plasma membrane transporters that are responsible for removal of chemicals, xenobiotic conjugates and oxidative stress products across the sinusoidal (e.g., Mrp1, 3, and 4) and canalicular (e.g., Mrp2) hepatocyte membranes. Levels of Mrp2, Mrp3, and Mrp4 mRNA and protein are elevated in mouse liver during APAP injury (Aleksunes *et al.*, 2005, 2006). Induction of Mrp3 and Mrp4 proteins occurs primarily in centrilobular hepatocytes (Aleksunes *et al.*, 2006). Human liver specimens from APAP cases also exhibit increased levels of MRP4 mRNA and protein (Barnes *et al.*, 2007).

Upregulation of efflux transporters may serve to limit the chemical burden of hepatocytes during critical stages of regeneration and repair following induction of acute liver injury. The purpose of this study was to study the relationship between Mrp3 and Mrp4 protein expression and compensatory proliferation of hepatocytes in a mouse model of APAP autoprotection. This is the first report of autoprotection in mice using a single pretreatment dose of APAP. Mrp3 and Mrp4 proteins were selected for analysis because they are strongly upregulated in mice after a single dose of APAP.

MATERIALS AND METHODS

Chemicals

APAP, propylene glycol, 10% zinc formalin, N-acetyl-cysteine (NAC) and reduced GSH were purchased from Sigma-Aldrich (St Louis, MO). All other reagents were of reagent grade or better.

Treatment Regimen

Male C57BL/6J mice, aged 10–12 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a 12-h dark/light cycle, temperature- and humidity-controlled environment and fed *ad libitum*.

Dosing regimen 1. Following overnight fasting, groups of mice ($n = 4$–8) were treated with 400 mg APAP/kg or vehicle (50% propylene glycol; 5 ml/kg

ip). Plasma and liver samples were collected at 24, 48, 72, and 96 h after treatment.

Dosing regimen 2. Following overnight fasting, groups of mice were treated with 400 mg APAP/kg or vehicle. Mice were challenged with a second dose of APAP (600 or 700 mg/kg) or vehicle 48 h later. Plasma and liver samples were collected 0, 0.5, 2, 4, 8, and 24 h after the second dose of APAP for analysis of hepatotoxicity, GSH content, protein expression of transporters and enzymes, immunohistochemistry, and *in vitro* microsomal bioactivation and glucuronidation of APAP.

Dosing regimen 3. To block hepatocyte proliferation, 2 mg/kg colchicine or vehicle (saline; 5 ml/kg ip) was administered to mice 24 h after pretreatment with 400 mg APAP/kg and 1 h after challenge with APAP (600 or 700 mg/kg). Plasma and liver samples were collected 24 h after the second APAP dose. All animal studies were conducted in accordance with National Institutes of Health standards and the *Guide for the Care and Use of Laboratory Animals*. Studies were approved by the Institutional Animal Care and Use Committee.

Alanine Aminotransferase Activity

Plasma alanine aminotransferase (ALT) activity was determined as a biochemical indicator of hepatocellular necrosis. Infinity ALT Liquid Stable Reagent (Thermotrace, Melbourne, Australia) was used according to the manufacturer's protocol.

Histopathology

Liver samples were fixed in 10% neutral-buffered zinc formalin prior to routine processing and paraffin embedding. Liver sections (5 μm) were stained with hematoxylin and eosin. Sections were examined by light microscopy for the presence and severity of necrosis and degeneration using a grading system described previously (Manautou *et al.*, 1994).

Preparation of Homogenate and Crude Membrane Fractions

Liver homogenate and plasma membrane preparations were made as described previously (Aleksunes *et al.*, 2006). Protein concentrations were determined using Bio-Rad protein assay reagents (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Proteins were electrophoretically resolved using polyacrylamide gels (8–12% resolving, 4% stacking) and transblotted overnight at 4°C onto polyvinylidene fluoride-Plus membrane (Micron Separations, Westboro, MA). Immunochemical detection of Cyp1a2, Cyp2e1, Cyp3a11, glutamate cysteine ligase catalytic (Gclc), glutamate cysteine ligase modifier (Gclm), UDP-glucuronosyltransferase 1a6 (Ugt1a6), Mrp3, Mrp4, heme oxygenase-1 (HO-1), and β-actin proteins are described in Supplementary Table 1 and as previously published (Aleksunes *et al.*, 2006). Protein-antibody complexes were detected using an enhanced chemiluminescent kit (Amersham Life Science, Arlington Heights, IL) and exposed to Fuji Medical X-ray film (Fisher Scientific, Springfield, NJ). Intensity of protein bands was quantified using the Discovery Series Quantity One 1-D Analysis software (Bio-Rad Laboratories, Hercules, CA).

In Vitro *Activation and Glucuronidation of APAP*

Liver microsomes were prepared 48 h after vehicle or APAP treatment of mice (400 mg/kg) as previously described (Manautou *et al.*, 1994, 1996). Microsomal activation of APAP to the reactive intermediate NAPQI was measured indirectly using NAC as a trapping agent. Hepatic Ugt activity toward APAP was also assessed. Formation of APAP-NAC and APAP-GLU was determined by high performance liquid chromatography. APAP-NAC and APAP-GLU concentrations were calculated by comparison of integrated peak areas to those of known concentrations of APAP, which has a similar extinction coefficient (Howie *et al.*, 1977). Activity is expressed as nmol/min/mg protein.

Nonprotein Sulfhydryl

Nonprotein sulfhydryl (NPSH) concentration was measured as an indicator of reduced GSH following the colorimetric procedure of Ellman (1959). NPSH concentration was quantified by comparison to a GSH standard curve.

Sections of formalin-fixed, paraffin-embedded livers were deparaffinized in xylene, then rehydrated through a graded alcohol series. For double immunohistochemical staining of Mrp4 with 4-hydroxynonenal (4-HNE) or proliferating cell nuclear antigen (PCNA), sections were incubated overnight with rat anti-Mrp4 antibody (M$_4$I-10) diluted 1:100. M$_4$I-10 antibody was generously provided by George Scheffer, VU Medical Center, Amsterdam, The Netherlands. Protein-antibody complexes were visualized using the mouse Vectastain Elite ABC kit and developed with 3,3′-diaminobenzidine according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Sections were then counterstained for 4-HNE or PCNA. ALX-210-767 (Axxora, San Diego, CA) antibody was diluted 1:500 and applied to slides for detection of 4-HNE staining. Protein-antibody complexes were visualized using the Vectastain Elite ABC kit (Vector Laboratories). PCNA was stained using the Zymed PCNA kit (Invitrogen, Carlsbad, CA). 4-HNE and PCNA immunostaining was visualized by developing with Vector VIP peroxidase substrate (Vector Laboratories, Burlingame, CA). Tissues were counterstained with methyl green, followed by dehydration in ethanol and clearing with xylene. Liver sections from three to six mice per group were stained and representative images are shown. Negative control staining was performed by incubating sections without primary antibody. Sections were imaged using a Nikon Optiphot microscope (Donsanto Corp., Natick, MA) equipped with a Spot Insight 2MP Firewire Color Mosaic Camera and Spot Software v4.5 (Diagnostic Instruments, Inc., Sterling Heights, MI).

Statistical Analysis

Quantitative results were expressed as means ± SE ($n = 3$–16 mice). Data were analyzed using unpaired *t*-test or one-way ANOVA followed by Newman-Keuls multiple range test. Histopathological data were rank ordered prior to ANOVA analysis. Significance was set at $p < 0.05$.

RESULTS

Time Course of Plasma ALT Activity after APAP Treatment

Administration of 400 mg/kg APAP to male C57BL/6J mice resulted in hepatic injury as determined by plasma ALT levels (dosing regimen 1, Fig. 1A). Elevated plasma ALT activity (175 U/l) was observed at 24 and 48 h after APAP (mean plasma ALT activity in control mice: 32 U/l). By 72 and 96 h, plasma ALT levels were not statistically different from controls.

Time Course of HO-1, Mrp3, and Mrp4 Protein Expression after APAP Treatment

Expression of HO-1, Mrp3, and Mrp4 proteins in response to APAP was measured by western blot (dosing regimen 1, Fig. 1B). HO-1 is a highly inducible stress protein which is responsible for catabolism of heme released from drug metabolizing enzymes during hepatocyte injury (Bauer and Bauer, 2002). We analyzed HO-1 protein levels after APAP treatment in order to compare temporal changes in the expression of transport proteins to a known indicator of the hepatic stress response. Representative blots are shown in Figure 1B. Expression of HO-1 protein was elevated 6.4- and 5.4-fold at 24 and 48 h after APAP treatment, respectively. HO-1 expression returned to control levels by 72 h (Fig. 1B).
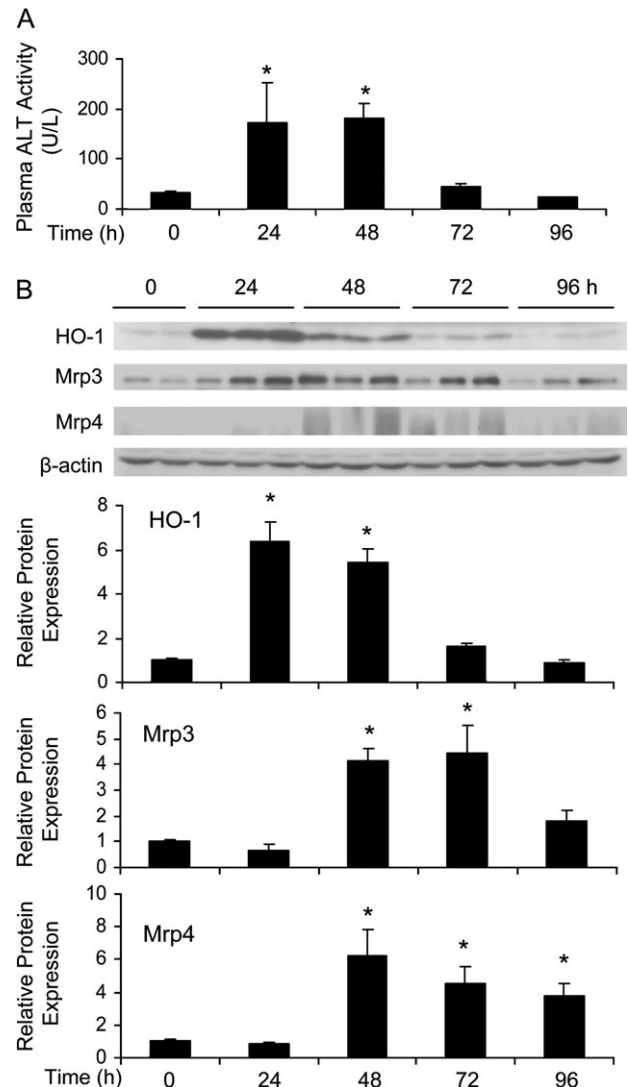
FIG. 1. Plasma ALT activity and HO-1, Mrp3, Mrp4 protein expression after APAP treatment. Plasma and livers were isolated from mice 24, 48, 72, and 96 h following APAP 400 mg/kg or vehicle administration. (A) The data are presented as mean plasma ALT activity (U/l) ± SE ($n = 4$–8 animals). (B) Western blots for HO-1, Mrp3, and Mrp4 were performed using liver membrane preparations from control and APAP-treated mice. Equal protein loading (50 μg protein/lane) was confirmed by detection of β-actin. Representative time course blots ($n = 2$–3 animals) are shown (upper panel). The data from individual Western blots (for each time point) are presented as mean relative protein expression ± SE ($n = 4$–6 animals) (lower panel). Asterisks (*) represent a statistical difference ($p < 0.05$) between pooled 0-h vehicle- and APAP-treated groups.

By contrast, increases in Mrp3 and Mrp4 proteins were not observed until 48 h. Exposure to APAP increased Mrp3 4.5-fold at 48 and 72 h. Upregulation of Mrp4 protein in mouse liver persisted through the 96-h time point with maximal induction (sixfold) at 48 h. These data demonstrate upregulation of mouse HO-1, Mrp3, and Mrp4 proteins at varying time points after APAP treatment.

Immunohistochemical Detection of 4-HNE and PCNA Proteins in Relation to Mrp4 after APAP Treatment

Double immunohistochemical staining was performed on paraffin liver sections from control and APAP-treated mice to study the localization of increased Mrp4 protein with respect to lipid peroxidation (4-HNE adduct staining) and tissue repair (PCNA staining) (dosing regimen 1, Figs. 2 and 3). Because the M$_3$II-2 antibody that detects mouse Mrp3 protein does not work on paraffin sections, immunohistochemical analysis of transporters was limited to Mrp4. Two magnifications are provided to highlight changes within the liver lobule (20X) and immediately around the central vein (40×). Mrp4, 4-HNE adducts and PCNA proteins were minimally detected in liver sections from control mice (Figs. 2A, 2B and Figs. 3A, 3B). Minimal PCNA staining was seen at 24 h (Figs. 3C and 3D). By contrast, strong cytoplasmic 4-HNE staining (*purple*) of centrilobular hepatocytes was observed in sections from APAP-treated mice at 24 h (Figs. 2C and 2D). A decrease in intensity of 4-HNE adducted proteins was seen at 48 h after APAP, with the appearance of pronounced basolateral Mrp4 staining (*brown*) in two to three layers of hepatocytes surrounding the central vein (Figs. 2E and 2F). At this time point also, PCNA-positive nuclei (*purple*) were first observed in liver sections from APAP-treated mice (Figs. 3E and 3F). Mrp4 and PCNA staining was maximal at 48 h and colocalized in centrilobular hepatocytes. By 72 and 96 h, no 4-HNE staining was detected and the intensity of Mrp4 staining and the number of PCNA-positive cells were reduced (Figs. 2G–J and 3G–J).

Effect of APAP Pretreatment on Plasma ALT Activity and Histopathology following APAP Challenge

Mice pretreated with either vehicle or 400 mg/kg APAP were challenged 48 h later with a higher dose of APAP (600 or 700 mg/kg). Forty-eight h was selected for APAP challenge because levels of Mrp3, Mrp4, and PCNA proteins were highest then (Fig. 1B and Figs. 3E/F). Plasma ALT activity was measured 24 h after APAP challenge (dosing regimen 2, Fig. 4). In vehicle-pretreated mice, dose-dependent increases in plasma ALT were observed after APAP challenge (600 mg/kg: 1780 U/l and 700 mg/kg: 3400 U/l). Pretreatment of mice with APAP significantly reduced plasma ALT levels following challenge with the higher doses of APAP (600 mg/kg: 260 U/l and 700 mg/kg: 1040 U/l). No mortality was observed in any treatment group.

Severity of hepatocellular lesions was analyzed and graded using a scale ranging from 0 to 5. Liver samples with grades greater than 2 are considered to have significant injury (Manautou *et al.*, 1994). Examination of liver sections from control mice that received two vehicle injections demonstrated normal histology (Table 1). Mice pretreated with APAP 400 mg/kg and challenged with vehicle also had normal liver histology. Thirty seven percent of vehicle-pretreated mice that were challenged with either 600 or 700 mg APAP/kg had significant liver injury. By contrast, pretreatment with 400 mg APAP/kg resulted in mild (score 1) to moderate (score 2) centrilobular degeneration after challenge with either 600 or 700 mg APAP/kg, with no animals exhibiting scores greater than 2.

Effect of APAP Pretreatment on Expression and Activity of Bioactivation and Detoxification Pathways

The contribution of altered APAP bioactivation and detoxification to autoprotection was assessed by measuring protein expression and function of critical metabolic enzymes after APAP pretreatment. Mice were given vehicle or APAP 400 mg/kg and sacrificed 48 h later. Mice were also fasted overnight prior to sacrifice to recreate conditions at the time of APAP challenge. Cyp1a2, 2e1, and 3a catalyze the formation of the APAP reactive intermediate NAPQI in rodent liver. Individual blots for Cyp1a2, 2e1, and 3a11 are shown in Figure 5A. Expression of these Cyp proteins was not altered by APAP pretreatment (Fig. 5B). The *in vitro* formation of NAPQI in microsomal preparations from vehicle- and APAP-pretreated livers was determined using NAC as a trapping agent (Fig. 5C). Formation of APAP-NAC was not changed by APAP treatment suggesting that changes in APAP bioactivation are not the reason for autoprotection.

APAP hepatotoxicity is dependent not only upon Cyp proteins, but also the expression and activity of detoxification-related enzymes. The majority of the parent APAP compound is conjugated to glucuronic acid and excreted as APAP-GLU. Ugt1a6 is the main Ugt isoform responsible for APAP-GLU formation (Kessler *et al.*, 2002). Expression of Ugt1a6 protein was unchanged in livers from APAP-pretreated mice (Figs. 6A and 6B). Accordingly, the *in vitro* formation of APAP-GLU in microsomal preparations was not changed by prior APAP treatment of mice suggesting that altered glucuronidation is not contributing to autoprotection (Fig. 6C).

NAPQI can be detoxified through nucleophilic sequestration by GSH. Gcl performs the rate-limiting step in GSH synthesis by catalyzing the formation of γ-glutamylcysteine from glutamine and cysteine in the presence of ATP. Gcl is a holoenzyme comprised of two subunits, a catalytic subunit (Gclc) and a modifier subunit (Gclm). Expression of Gclc and Gclm proteins was 1.2-fold and 1.5-fold higher in APAP-pretreated mice, respectively (Figs. 6A and 6B). Levels of reduced GSH at 48 h after APAP were also measured indirectly by quantification of total hepatic NPSH in the liver. Despite the increases in hepatic Gclc and Gclm proteins, the amount of NPSH in livers from APAP-pretreated mice was not different from that in their vehicle-pretreated counterparts (Fig. 6D). In response to challenge with APAP 600 mg/kg (dosing regimen 2), there were similar declines in NPSH at 0.5, 2, and 4 h in both vehicle- and APAP-pretreated mice. However, by 8 and 24 h after challenge, toxicant-pretreated mice demonstrated

20X   40X
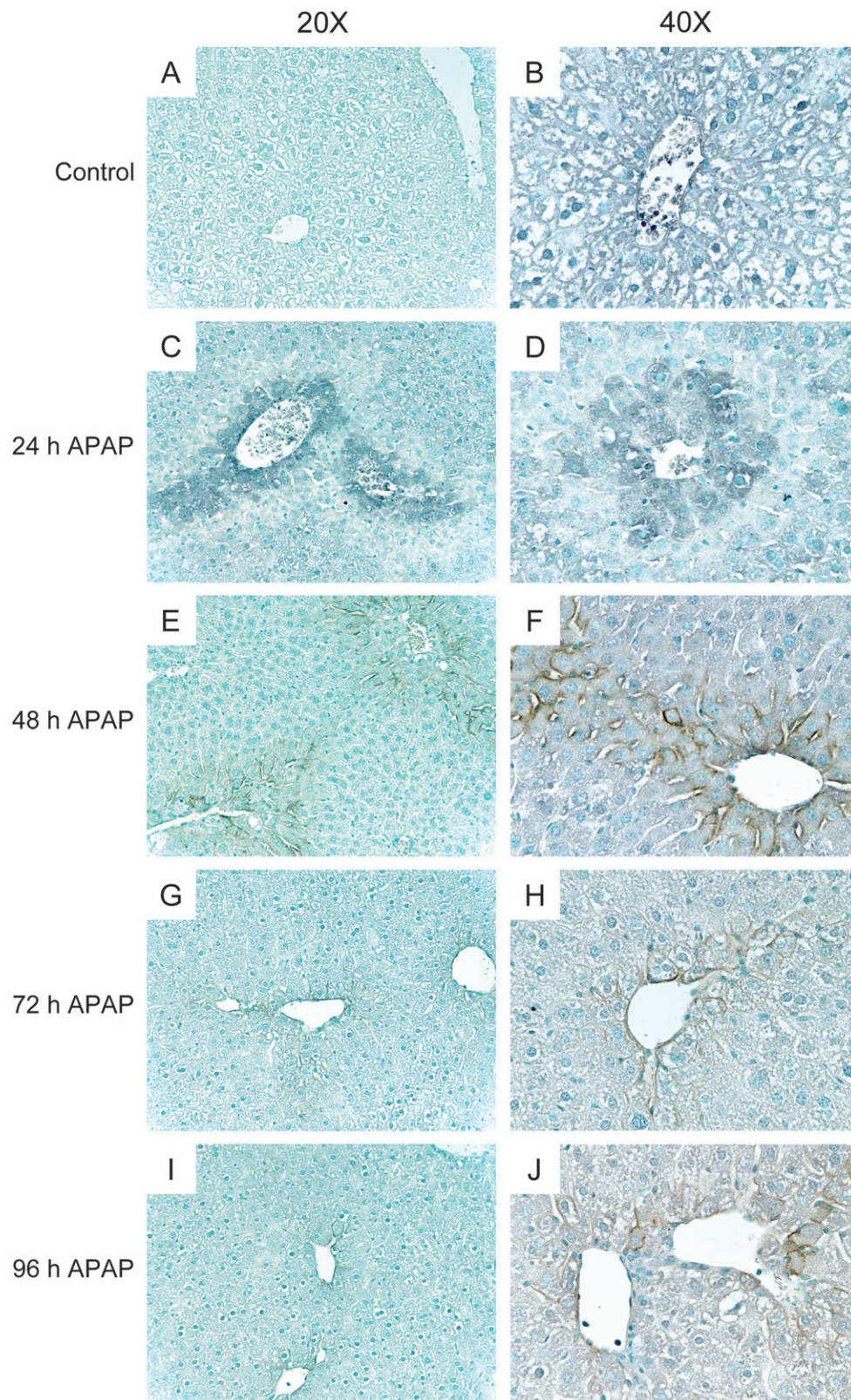
FIG. 2. Immunohistochemical detection of 4-HNE in relation to Mrp4 after APAP treatment. Immunohistochemical staining to detect 4-HNE (purple) and Mrp4 (brown) was conducted on paraffin-embedded liver sections from control and APAP 400 mg/kg-treated mice at 24, 48, 72, and 96 h. Representative images are shown at two magnifications (20× and 40×). (A/B) Control; (C/D) APAP-treated 24 h; (E/F) APAP-treated 48 h; (G/H) APAP-treated 72 h; (I/J) APAP-treated 96-h liver sections.
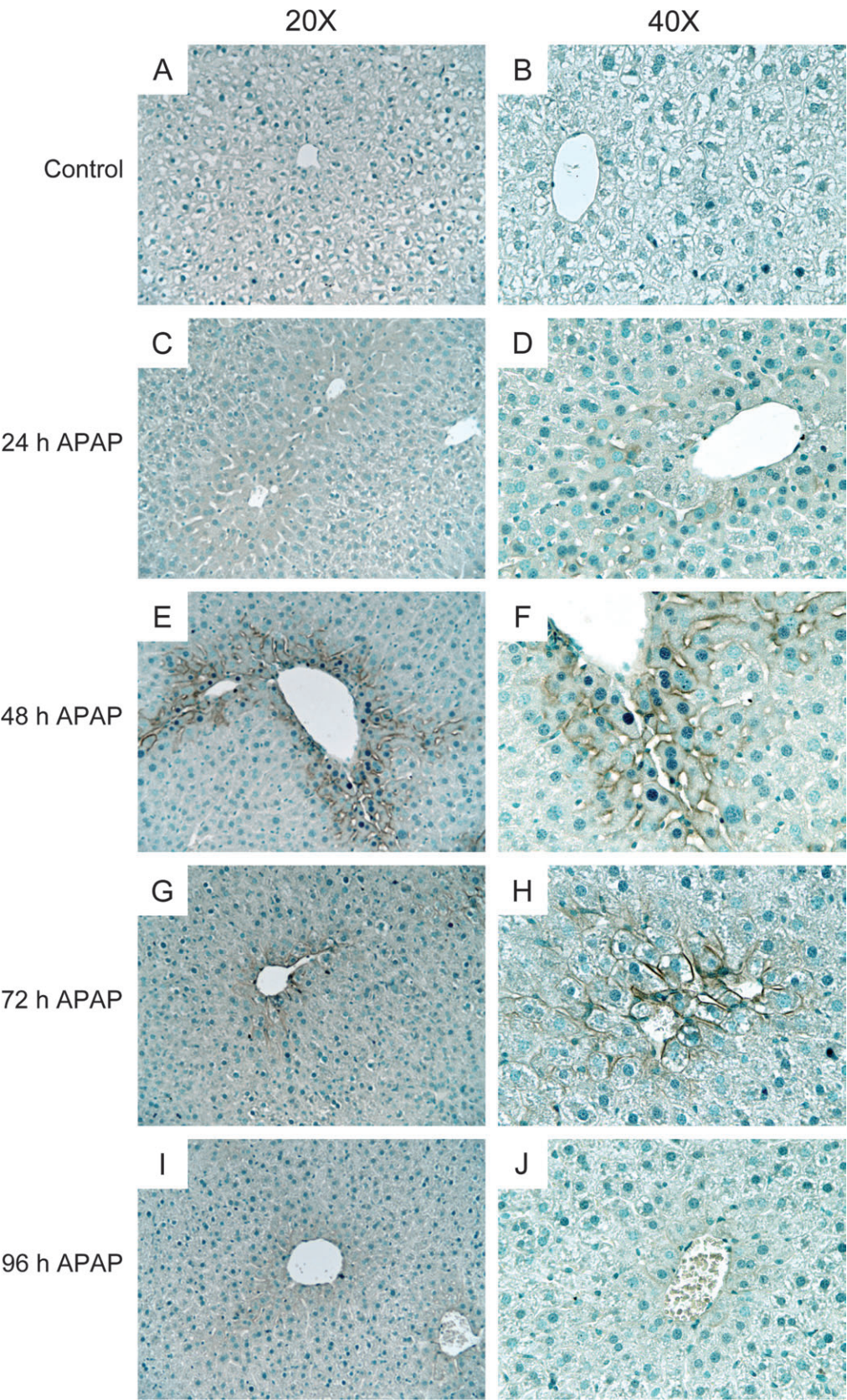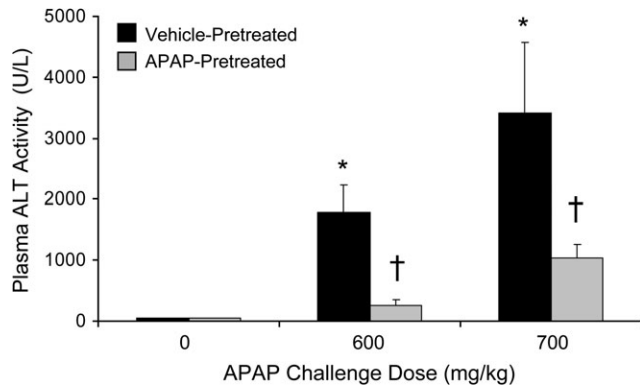
FIG. 4. Plasma ALT activity after APAP challenge in vehicle- and APAP-pretreated mice. Groups of mice were treated with vehicle or APAP 400 mg/kg. Forty-eight h later mice were challenged with vehicle or APAP (600 or 700 mg/kg). Plasma was isolated from mice 24 h following APAP challenge. The data are presented as mean plasma ALT activity (U/l) ± SE ($n = 4$–8 animals). Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle- and APAP-treated groups. Daggers (†) represent a statistical difference between vehicle-pretreated, APAP-challenged and APAP-pretreated, APAP-challenged groups.

TABLE 1
Histopathological Analysis of Livers from Vehicle- and APAP-Pretreated Mice after APAP Challenge

Treatment group	Histological grade						Percent > 2
	0	1	2	3	4	5	
Control/Control	4	0	0	0	0	0	0
APAP 400/Control	4	0	0	0	0	0	0
Control/APAP 600*	0	2	3	3	0	0	37.5
APAP 400/APAP 600*·†	0	7	1	0	0	0	0
Control/APAP 700*	0	1	4	2	1	0	37.5
APAP 400/APAP 700*,†	0	0	8	0	0	0	0

Note. Groups of mice received vehicle (control) or APAP (400 mg/kg: APAP 400). A second dose of APAP (600 or 700 mg/kg: APAP 600, APAP 700) or vehicle was administered 48 h later. Livers were removed from mice 24 h following the challenge dose and fixed in 10% formalin prior to paraffin embedding and staining with hematoxylin and eosin. Liver slices were evaluated for the severity of degenerative and necrotic changes in centrilobular regions as described previously (Manautou *et al.*, 1994). Liver samples with grades greater than 2 are considered to have significant injury. Data were rank ordered prior to statistical analysis. Asterisks (*) represent a statistical difference ($p < 0.05$) from Control/Control mice. Daggers (†) represent a statistical difference between vehicle-pretreated, APAP-challenged and APAP-pretreated, APAP-challenged groups.

higher recovery of NPSH in comparison to vehicle-pretreated mice, which correlated with lower plasma ALT activity in these mice (Figs. 7A and 7B).

Effect of APAP Pretreatment on HO-1, Mrp3, and Mrp4 Protein Expression following APAP Challenge

Because the expression of detoxification enzymes and transporters is induced during APAP hepatotoxicity, HO-1, Mrp3, and Mrp4 proteins were measured in livers 24 h following APAP (600 mg/kg) challenge from vehicle- and APAP-pretreated mice (Dosing regimen 2, Fig. 8B). Individual blots are shown in Figure 8A. HO-1 levels were induced 3.4-fold by APAP (600 mg/kg) in vehicle- and APAP-pretreated mice compared to double vehicle mice. Mrp3 protein expression was unchanged in all treatment groups. This is not unexpected because upregulation of Mrp proteins after APAP hepatotoxicity typically occurs at 48 h and later (Figs. 1B and 1C) (Aleksunes *et al.*, 2006). By contrast, a robust increase in Mrp4 protein (10-fold) was observed at 24 h after APAP challenge only in APAP-pretreated mice (Fig. 8B). In addition, mice which received APAP pretreatment and were challenged with vehicle had slightly increased Mrp4 protein (twofold), although this was not statistically significant.

Effect of APAP Pretreatment on Immunohistochemical Detection of Mrp4 and PCNA Proteins following APAP Challenge

Double immunohistochemical staining for Mrp4 and PCNA was performed on paraffin liver sections. Two magnifications are provided to highlight changes within the liver lobule (20×) and immediately around the central vein (40×). No staining for Mrp4 or PCNA was seen in control mice (Figs. 9A and 9B). Mice that received only the APAP 400 mg/kg pretreatment, but no APAP challenge, had basolateral Mrp4 (*brown*) and nuclear PCNA (*purple*) staining in centrilobular hepatocytes (Figs. 9C and 9D). Liver sections from vehicle-pretreated, APAP-challenged mice exhibited some PCNA staining in hepatocytes at the periphery of degenerate and necrotic cells (Figs. 9E and 9F). No Mrp4 staining was observed in these tissues. By contrast, staining for Mrp4 and PCNA was very strong and colocalized in centrilobular hepatocytes of APAP-pretreated, APAP-challenged mice (Figs. 9G and 9H).

In addition, APAP-pretreated, APAP-challenged mice also exhibit marked vacuolation in hepatocytes suggestive of glycogen accumulation (Figs. 9G and 9H), which was not

FIG. 3. Immunohistochemical detection of PCNA in relation to Mrp4 after APAP treatment. Immunohistochemical staining to detect PCNA (purple) and Mrp4 (brown) was conducted on paraffin-embedded liver sections from control and APAP 400 mg/kg-treated mice at 24, 48, 72, and 96 h. Representative images are shown at two magnifications (20× and 40×). Panels (A/B) control; (C/D) APAP-treated 24 h; (E/F) APAP-treated 48 h; (G/H) APAP-treated 72 h; (I/J) APAP-treated 96-h liver sections.
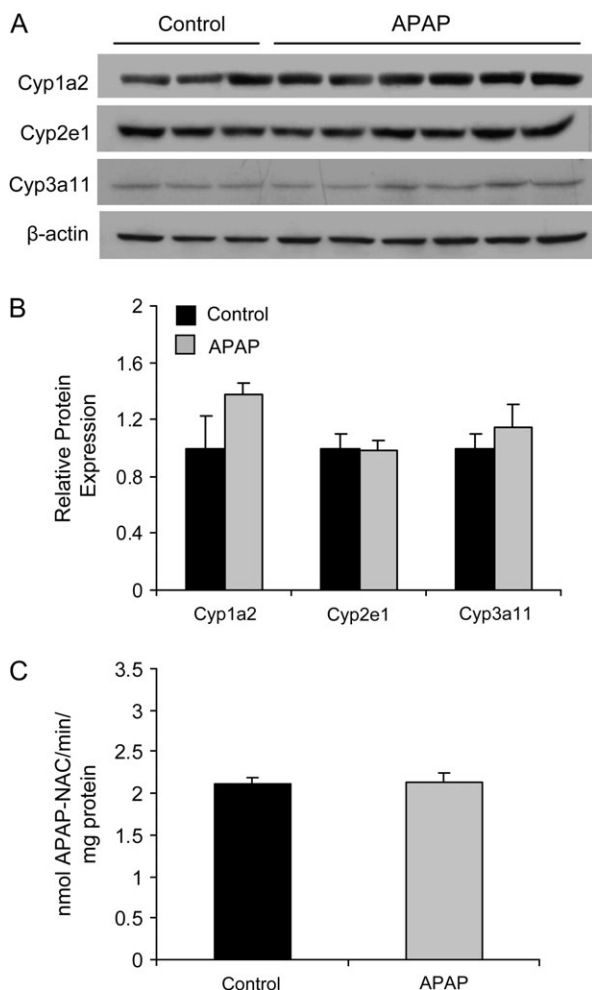
FIG. 5. Bioactivation pathways for APAP in livers from vehicle- and APAP-pretreated mice. Western blots for Cyp1a2, Cyp2e1 and Cyp3a11 proteins were performed using homogenate preparations 48 h after vehicle (Control) or APAP 400 mg/kg treatment. The data are presented as individual blots (A) and as mean relative protein expression ± SE ($n = 3$–6 animals) (B). Equal protein loading (50 μg protein/lane) was confirmed by detection of β-actin. (C) Microsomes were prepared and assayed for APAP bioactivation as described in "Materials and Methods" ($n = 4$ animals). Data are presented as nmol APAP-NAC/min/mg protein. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and APAP-treated groups.

evident in vehicle-pretreated, APAP-challenged mice (Figs. 9E and 9F).

Effect of Colchicine Antimitotic Intervention on Plasma ALT Activity in APAP Autoprotected Mice

Previous reports demonstrate that hepatocyte proliferation is critical for conferring auto- and heteroprotection (Chanda et al., 1995; Shayiq et al., 1999). To determine the contribution of proliferation to this model of autoprotection, mice were administered two doses of colchicine (2 mg/kg); one at 24 h after APAP (400 mg/kg) pretreatment and the second at 1 h after APAP (600 mg/kg) challenge (dosing regimen 3).

Colchicine blocks proliferation by interfering with microtubule function. In addition, colchicine prevents S-phase DNA synthesis by inhibiting thymidine kinase and thymidylate synthetase activities (Tsukamoto and Kojo, 1989). This dose of colchicine is not hepatotoxic (Sawant et al., 2006). Plasma ALT activity was evaluated 24 h after challenge with the second dose of APAP. Of note, the first dose of colchicine did not alter plasma ALT levels at 48 h, when the APAP challenge is normally given (data not shown). Vehicle-pretreated mice had elevation of plasma ALT (910 U/l) after APAP challenge compared to control mice (Fig. 10A). Pretreatment of mice with APAP 400 mg/kg significantly reduced the plasma ALT level to 157 U/l following APAP 600 mg/kg challenge. APAP-pretreated mice administered colchicine resulted in plasma ALT levels of 832 U/l, demonstrating that colchicine blocked APAP autoprotection. Of note, one mouse in the APAP 400/APAP 600/colchicine group died.

Effect of Colchicine Antimitotic Intervention on Mrp4 Protein Expression in APAP Autoprotected Mice

The effect of colchicine on Mrp4 induction during autoprotection was also evaluated. Mrp4 protein levels in APAP-pretreated, APAP-challenged mice were increased 14-fold at 24 h after APAP challenge (Fig. 10B). Livers from APAP-pretreated mice given colchicine showed remarkably lower levels of Mrp4 protein expression after toxicant challenge, suggesting that colchicine interfered not only with hepatocyte proliferation, but also Mrp4 induction.

DISCUSSION

In the present study, we investigated the hepatic expression of Mrp3 and Mrp4 proteins in a mouse model of APAP autoprotection. A time course study revealed lipid peroxidation, hepatocyte injury, as well as compensatory induction of the stress protein HO-1 at 24 h after a single dose of APAP. Maximal induction of Mrp3 and Mrp4 proteins occurred by 48 h and levels of both transporters remained elevated through 72 (Mrp3) and 96 (Mrp4) h. Overexpression of Mrp4 in livers from APAP-treated mice occurred in proliferating centrilobular hepatocytes. APAP pretreatment protected mice given a second, higher dose of APAP 48 h later. Expression of Cyp1a2, 2e1, 3a11, and Ugt1a6 proteins as well as in vitro formation of APAP-NAC and APAP-GLU in livers from APAP-pretreated mice was not different from vehicle-pretreated controls. Gclc and Gclm proteins were increased in APAP-pretreated livers however these changes did not alter basal levels of GSH. Instead, GSH recovery was greater in livers from APAP-pretreated mice as evidenced by higher NPSH levels at 8 and 24 h, likely contributing to autoprotection. Expression of HO-1, Mrp3, and Mrp4 proteins in response to the second, higher dose of APAP was measured. HO-1 was similarly induced in
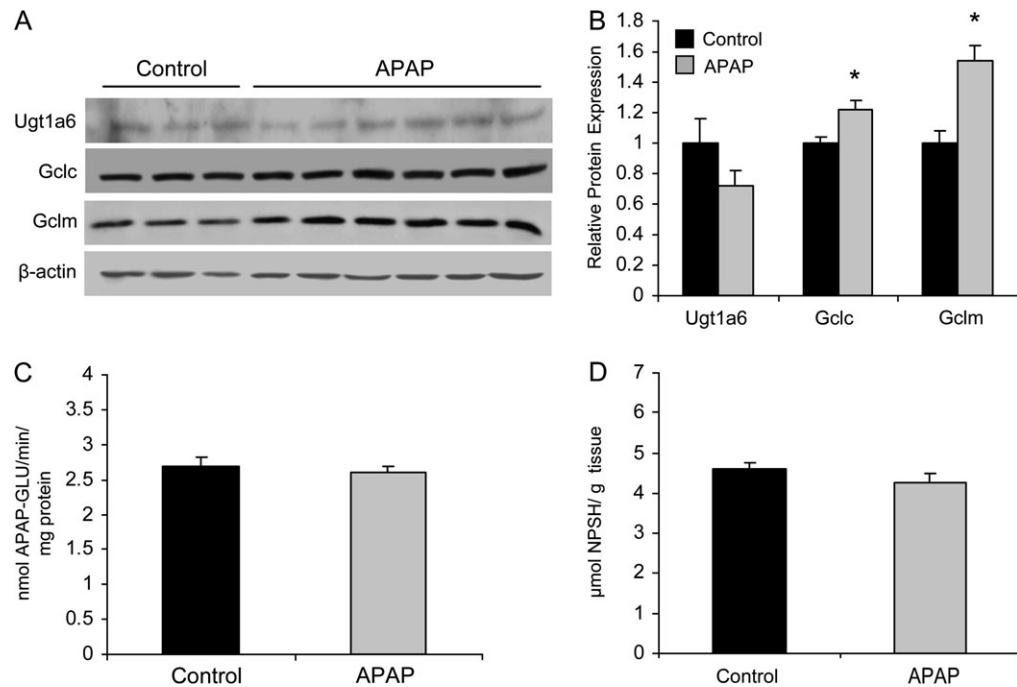
FIG. 6. Detoxification pathways for APAP in livers from vehicle- and APAP-pretreated mice. Western blots for Ugt1a6, Gclc, and Gclm proteins were performed using homogenate preparations 48 h after vehicle (Control) or APAP 400 mg/kg treatment. The data are presented as individual blots (A) and as mean relative protein expression ± SE (B). Equal protein loading (50 µg protein/lane) was confirmed by detection of β-actin. (C) Microsomes were prepared and assayed for APAP glucuronidation as described in Materials and Methods. Data are presented as nmol APAP-GLU/min/mg protein. (D) Basal NPSH levels expressed as µmol NPSH/g tissue. Asterisks (*) represent a statistical difference ($p < 0.05$) between control and APAP-treated groups.

both vehicle- and APAP-pretreated mice after APAP challenge. No changes in Mrp3 protein expression were observed in any of the groups. This is in contrast to Mrp4 which was markedly induced (10-fold) in mice that received both APAP pretreatment and challenge. Induction of Mrp4 in these mice colocalized to regions of hepatocyte proliferation. In order to further investigate the relationship between Mrp4 and proliferation, colchicine was introduced into the dosing regimen. Colchicine not only blocked APAP autoprotection, but it also abolished induction of Mrp4.

There is clinical data suggesting that humans can adapt to APAP-induced hepatotoxicity similar to our observations in rodents. In describing the incremental dose model of tolerance development to APAP hepatotoxicity in mice, Shayiq et al. (1999) introduced a clinical case in which a physician reported developing tolerance to chronic abuse of a prescription opiod/APAP combination product. More recently, a randomized, single-blind, placebo-controlled study revealed ALT elevations in a subset of healthy adults given 4 g APAP daily over a 14-day period (Watkins et al., 2006). ALT elevations tended to peak around 7–8 days with decrease by the 14th day suggesting that hepatocytes in these patients adapted to mild injury during repeat exposure to APAP. This clinical evidence clearly supports preclinical evaluation of mechanisms underlying APAP autoprotection. Pilot studies in our laboratory demonstrate induction of Mrp3 and Mrp4 proteins in mice treated

with incremental doses of APAP. This observation further validates changes observed in the current study using a single dose of APAP and may explain the findings of Shayiq et al. (1999).
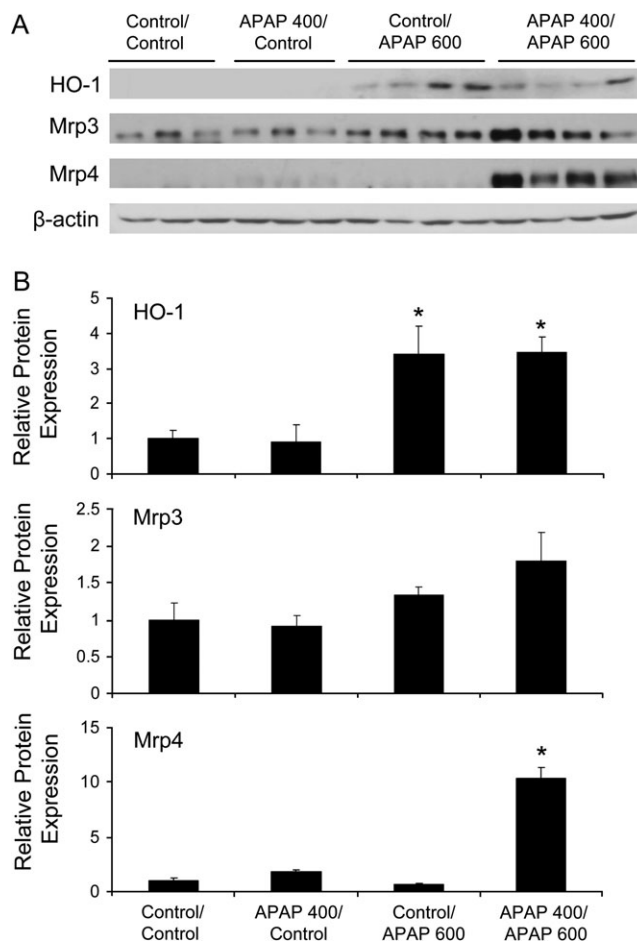
Upregulation of Mrp3 and Mrp4 could contribute to autoprotection in two ways. Ghanem et al. (2005) proposes that increases in Mrp3 protein may enhance the vectorial excretion of APAP-GLU across the sinusoidal membrane during autoprotection. Early work using chemical inducers and genetic models of Mrp3 induction demonstrated enhanced plasma levels of APAP-GLU in rats (Chen et al., 2003; Slitt et al., 2003; Xiong et al., 2002). In addition, sinusoidal excretion of APAP-GLU is reduced in transgenic mice lacking Mrp3 (Manautou et al., 2005; Zamek-Gliszczynski et al., 2006). Experiments in this study similarly demonstrated elevations in Mrp3 protein and activity which were reflected in higher plasma and urinary APAP-GLU concentrations in APAP-pretreated mice (data not shown). However, APAP-GLU is not considered to be a toxic metabolite. It can be speculated that a more efficient removal of APAP conjugates may prevent their accumulation and likelihood for causing substrate inhibition of detoxifying pathways. However, it remains unclear how enhanced APAP-GLU transport would explain APAP autoprotection (Ghanem et al., 2005). We propose that Mrps, in particular Mrp3 and Mrp4, may confer protection against APAP toxicity by transporting byproducts of cell injury. Mrp4
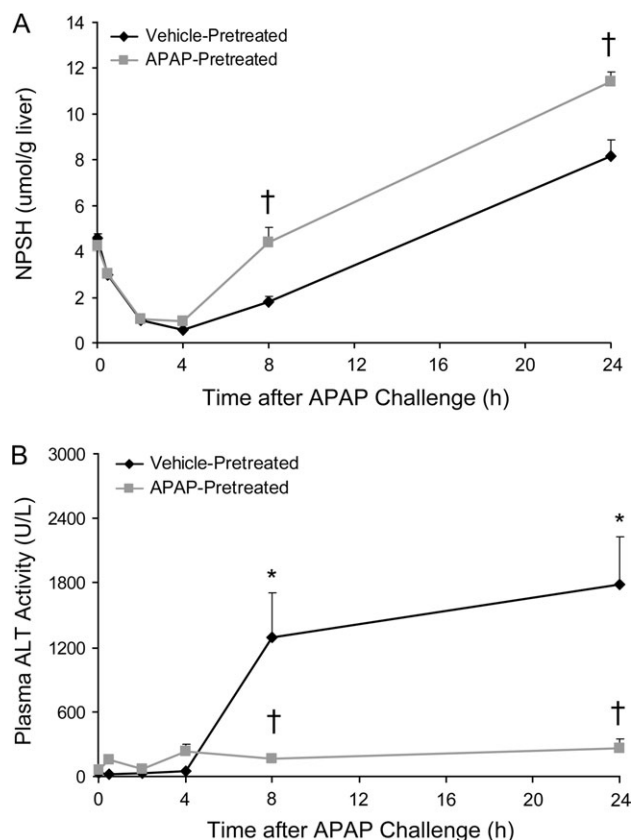
FIG. 7. GSH recovery in livers after APAP pretreatment and challenge. Plasma and liver were collected from vehicle- and APAP-pretreated mice at 0.5, 2, 4, 8, and 24 h after APAP 600 mg/kg challenge. (A) The data are presented as mean NPSH (μmol/g liver) $\pm$ SE ($n = 4$ animals). (B) The data are presented as mean plasma ALT activity (U/l) $\pm$ SE ($n = 4$ animals). Asterisks (*) represent a statistical difference ($p < 0.05$) between vehicle- and APAP-treated groups. Daggers ($\dagger$) represent a statistical difference between vehicle-pretreated, APAP-challenged and APAP-pretreated, APAP-challenged groups.
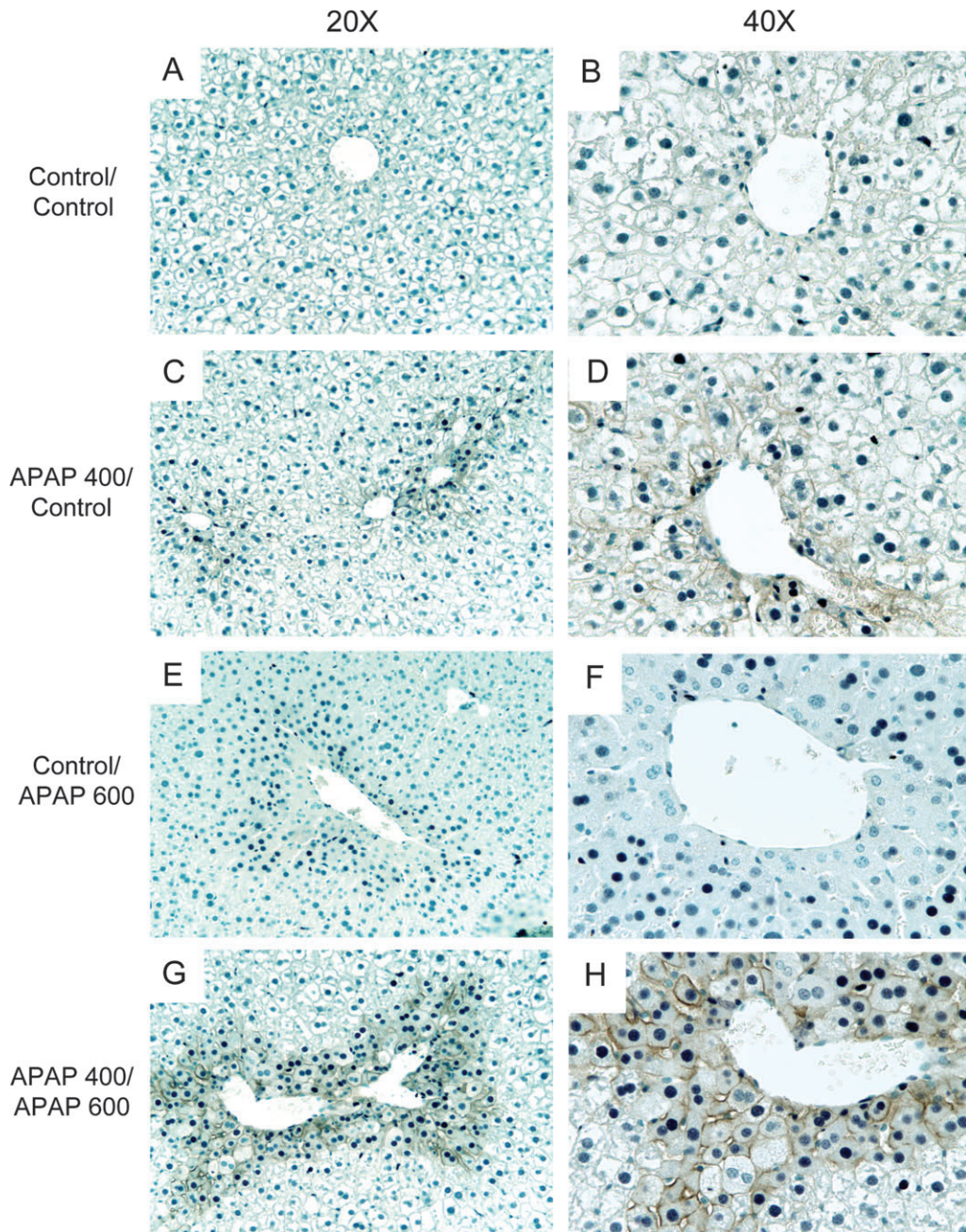

FIG. 8. HO-1, Mrp3, and Mrp4 protein expression after APAP pretreatment and challenge. Groups of mice were treated with vehicle (Control) or APAP (400 mg/kg: APAP 400). Forty-eight hours later mice were challenged with vehicle (Control) or APAP (600 mg/kg: APAP 600). Livers were isolated from mice 24 h following APAP challenge. Western blots for HO-1, Mrp3, and Mrp4 were performed using liver membrane preparations. Equal protein loading (50 μg protein/lane) was confirmed by detection of β-actin. (A) The data are presented as representative blots ($n = 3$) and (B) as mean relative protein expression $\pm$ SE ($n = 3$). Asterisks (*) represent a statistical difference ($p < 0.05$) from Control/Control mice.

substrates include bile acids, cyclic nucleotides (cyclic adenosine monophosphate and cyclic guanosine monophosphate) and prostaglandins (PGE$_1$ and PGE$_2$) (Bai *et al.*, 2004; Chen *et al.*, 2001; Reid *et al.*, 2003; Rius *et al.*, 2006; Zelcer *et al.*, 2003). Clearance of these substrates via Mrps may limit the chemical burden of hepatocytes during periods of stress and/or recovery.

Our data support a role for Mrp transporters in APAP autoprotection. However, it is well-known that similar chemical resistance can be observed with other chemicals (i.e., CCl$_4$) (Pound and Lawson, 1975). CCl$_4$ hepatotoxicity induces Mrp1, Mrp2, and Mrp4 mRNA and protein in mouse liver (Aleksunes *et al.*, 2005, 2006). Studies by Page and Carlson found negligible biliary excretion of CCl$_4$ in rats suggesting transport-independent hepatic clearance (Page and Carlson, 1994). In cases of CCl$_4$ auto- or heteroprotection, Mrp transport of byproducts or mediators of cell stress may be more important to the resistant phenotype than altered disposition of the toxicant itself or its metabolites. CCl$_4$-mediated liver injury

is generally characterized by altered redox status and lipid peroxide formation. Mrp1 and Mrp2, which are induced in response to CCl$_4$ toxicity, can transport the lipid peroxide 4-HNE and may be important in protection against subsequent CCl$_4$ exposure (Ji *et al.*, 2002; Reid *et al.*, 2003).

One important feature of hepatocyte proliferation in models of autoprotection and heteroprotection is the faster and more robust initiation of cell division that occurs following toxicant re-exposure (Chanda *et al.*, 1995; Shayiq *et al.*, 1999). The initial pretreatment dose primes hepatocytes to enter the cell cycle and divide. Similarly, our data demonstrate a greater extent of proliferating hepatocytes at 24 h after APAP challenge in APAP-pretreated mice. Furthermore, induction of Mrp4 at this time point is earlier and of greater magnitude

FIG. 9. Immunohistochemical detection of Mrp4 and PCNA after APAP pretreatment and challenge. Groups of mice were treated with vehicle (Control) or APAP (400 mg/kg: APAP 400). Forty-eight hours later mice were challenged with vehicle (Control) or APAP (600 mg/kg: APAP 600). Livers were isolated from mice 24 h following APAP challenge. Immunohistochemical staining to detect PCNA (purple) and Mrp4 (brown) was conducted on paraffin-embedded liver sections. Representative images are shown at two magnifications (20× and 40×). Panels (A/B) Control/Control; (C/D) APAP 400/Control; (E/F) Control/APAP 600; (G/H) APAP 400/APAP 600 liver sections.

(10- to 14-fold) compared to a sixfold to eightfold increase in Mrp4 levels usually seen at 48 h after a single, lower dose of APAP. Our laboratory recently demonstrated that induction of Mrp4 and Gclc mRNA and protein in response to APAP is absent in Nrf2-null mice (Aleksunes *et al.*, 2008). In addition, Nrf2 is critical for liver regeneration in response to partial hepatectomy (Beyer *et al.*, 2008). Collectively, these studies suggest Nrf2 signaling is activated during autoprotection leading to rapid proliferation of hepatocytes that overexpress Gclc, Gclm and Mrp4. Coordinated upregulation of GSH and transport pathways may be one means for conferring resistance to chemical injury.
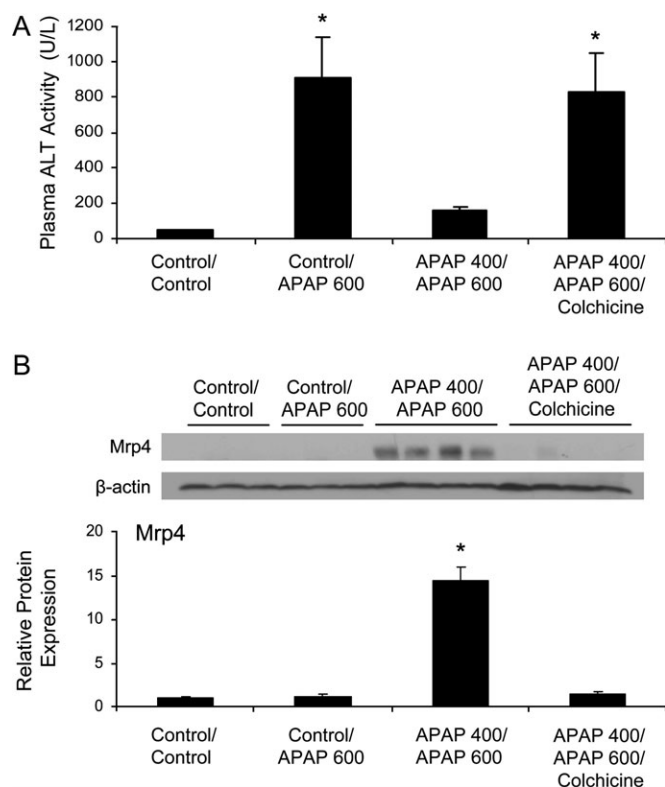
FIG. 10. Plasma ALT activity and Mrp4 protein expression after colchicine intervention in APAP autoprotected mice. Groups of mice were treated with vehicle (Control) or APAP (400 mg/kg: APAP 400). Colchicine (2 mg/kg) was administered at 24 and 49 h after APAP 400 mg/kg. Mice were challenged with vehicle (Control) or APAP (600 mg/kg: APAP 600) at 48 h. Plasma and livers were isolated from mice 24 h following APAP challenge. (A) The data are presented as mean plasma ALT activity (U/l) ± SE (n = 4–8 animals). (B) Western blot for Mrp4 was performed using liver membrane preparations. Equal protein loading (50 μg protein/lane) was confirmed by detection of β-actin. Asterisks (*) represent a statistical difference ($p < 0.05$) from Control/Control mice.

In conclusion, we have demonstrated that treatment of mice with APAP leads to induction of Gclc, Gclm, Mrp3, and Mrp4 proteins that correlates with a significant reduction in hepatotoxicity following challenge with a higher dose of APAP. In mice developing tolerance to APAP toxicity, upregulation of Mrp4 protein occurs preferentially in proliferating hepatocytes around the central vein. Antimitotic intervention with colchicine blocks autoprotection and Mrp4 upregulation, suggesting that induction of this transporter may be of mechanistic importance to chemoresistance in these cells. Increased basolateral excretion of chemicals to sinusoidal blood via Mrp3 and Mrp4 may serve not only as a protective mechanism for the efficient elimination of cytotoxic intermediates but also for paracrine signaling to adjacent hepatocytes and other cell types (including Kupffer and stellate cells) involved in progression of and/or recovery from liver disease.

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